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Subzero 12-hour Nonfreezing Cryopreservation of Porcine Heart in a Variable Magnetic Field

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Background. A novel subzero nonfreezing heart preservation method has been developed. It uses a refrigerating device that generates a variable magnetic field, allowing the whole organ to be cooled simultaneously to a supercooled state without the use of cryoprotectant. As a fundamental experiment for heart preservation, we verified whether this novel method is able to suppress anaerobic metabolism and reduce damage in the hearts of large animals. **Methods.** Twelve porcine hearts were collected and preserved for 12 hours using a simple immersion method. The hearts were divided into 2 groups: 6 underwent nonfreezing preservation at –3°C in a variable magnetic field (subzero group), and 6 underwent conventional preservation at 4°C (conventional group). The quantity of anaerobic metabolism and the degree of ultrastructural change in the 2 groups were evaluated and compared. **Results.** The concentration of adenosine triphosphate in the myocardial tissue was significantly greater in the subzero group than in the conventional group (21.06±5.87 μmol/g vs 5.96±3.41 μmol/g; $P < 0.05$). The accumulated lactate concentration was significantly lower in the subzero group than in the conventional group (6.58±2.28 μmol/g vs 11.15±3.74 μmol/g; $P < 0.05$). The Flameng score, an index of ultrastructural changes to the mitochondria, was significantly lower in the subzero group than in the conventional group (1.28±0.40 vs 2.73±0.30; $P < 0.05$). **Conclusions.** Subzero nonfreezing preservation using a variable magnetic field resulted in a remarkable suppression of anaerobic metabolism and myocardial protection in porcine hearts.

(*Transplantation* 2015;1: e33; doi: 10.1097/TXD.0000000000000544. Published online 9 October 2015)

In the field of heart transplantation, the time limit of heart preservation limits the donor pool; thus, development of a new method that allows longer preservation time is desired. The simple immersion method at 4°C is a simple, inexpensive, and reliable method for preserving donor hearts and has been the standard technique for heart preservation in current clinical practice. However, this conventional method is imperfect because anaerobic metabolism continues within the heart during preservation. The acceptable preservation time for a donor heart is limited to 6 hours; moreover, ischemic durations longer than 200 minutes have been associated with an increased mortality of transplant recipients.[1-3]

Many heart preservation methods are reportedly superior to the conventional simple immersion method at 4°C. New preserving solutions, new perfusion preservation methods, and subzero static preservation methods have been investigated. Although several preservation solutions have been developed, compared to the University of Wisconsin solution, no solution has shown a beneficial effect in clinical heart preservation since the 1980s.[3-6] Some perfusion preservation methods have been found to improve donor heart preservation in the hearts of both small and large animals.[7-12] However, because of the complexity and expense of the apparatus and the side effect of myocardial edema, perfusion preservation may not be suitable for clinical use.

Subzero preservation has also been investigated for the preservation of hearts. The principles of heart preservation involve the suppression of anaerobic metabolism due to ischemia, maintenance of spare energy for recovering cardiac function, and prevention of myocardial damage accompanying tissue acidosis.[13] Theoretically, lowering the preservation temperature by 10°C would lower the cellular metabolic demand by approximately 50%[13]; therefore, the lower the temperature, the greater the decrease in tissue metabolism. However, cellular damage due to coagulation and defrosting is inevitable at temperatures below the solidification point.[14,15] Thus, the temperature limit for adequate heart preservation has been determined to be 0 to 4°C. Studies have been performed for preserving an organ at a subzero temperature without freezing—the so-called

Received 21 July 2015.

Accepted 16 August 2015.

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This study was funded by the Department of General and Cardiothoracic Surgery, Kanazawa University.

The authors declare no conflicts of interest.

R.S. participated in the research design, the writing of the paper, the performance of the research, and data analysis. G.W. participated in research design and the writing of the article. H.K. participated in the writing of the article. S.Y. participated in research design and the performance of the research.

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ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000000544

supercooled state. Others have achieved subzero nonfreezing preservation by adding cryoprotectant into the cardioplegic solution, with promising results.[15-17] However, cryoprotectants in the solution mainly affect the surface of the cardiac tissue and are therefore not suitable for the preservation of large organs. Hence, the subzero nonfreezing preservation method has only been applied to the hearts of small animals. Moreover, because the precise mechanism of the manner in which cryoprotectants maintain the supercooled state is unknown, the toxicity of the cryoprotectants to the myocardium has been a matter of concern.

To develop a simple, cost-effective, nontoxic, and safe preservation method that is applicable to human hearts, and to overcome the limitations of heart preservation, we focused on a new technology that achieves the subzero nonfreezing state without the use of cryoprotectants. A Japanese company involved in the development of food-freezing technology invented a special refrigerator, termed as the Cells Alive System (CAS; ABI Co. Ltd., Chiba, Japan). Through the application of a combination of multiple weak energy sources, this refrigerator generates a special variable magnetic field that causes water molecules to oscillate, thus inhibiting crystallization during ice formation[18] (Figure 1). Because the entire material is frozen without the movement of water molecules, cells can be maintained intact and free of membranous damage. This refrigerator has the ability to achieve a nonfreezing state even below the solidifying point.

We are currently developing a subzero nonfreezing heart preservation method by simple immersion, using CAS. In CAS, it is possible to cool every tissue simultaneously to a subzero temperature and to maintain supercooled state. Reperfusion experiments using small animals (rats), conducted by Kato et al[18] in our institute, have demonstrated the positive effects of CAS in preserving cardiac function and suppressing metabolism. Before the novel preservation method can be put to clinical use, further evaluations are necessary using the hearts of large animals, which resemble those of human beings.

In the present study, we evaluated the effect of the CAS subzero nonfreezing method in preserving porcine hearts for a duration of 12 hours. As a fundamental experiment before heart transplantation, we aimed to verify whether this novel method is capable of suppressing anaerobic metabolism and tissue damage in the hearts of large animals.

MATERIALS AND METHODS

Twelve pigs weighing 27 to 36 kg were studied. All animals were cared for according to the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources of the National Research Council, published by the National Academy Press, revised in 2010. After anesthesia was induced by intramuscular injection of 10 mg/kg of ketamine hydrochloride, the pigs were placed in a dorsal position. Pancuronium bromide 0.1 mg/kg was injected intravenously for neuromuscular blockade. Tracheotomy was then performed, and controlled respiration was applied using an artificial respirator. Halothane inhalation was provided to maintain anesthesia. After a median sternotomy, 5 000 units of heparin were administered transvenously. The aorta was cross-clamped, and 1000 mL of University of Wisconsin Solution (Viaspan; Astellas Pharma Inc., Tokyo, Japan; containing potassium lactobionate 100 mM, KH_2PO_4 25 mM, $MgSO_4$ 5 mM, raffinose 30 mM, adenosine 5 mM, glutathione 3 mM, allopurinol 1 mM and hydroxyethyl starch 50 g/L) at 4°C was injected antegradely from the aortic root to induce immediate cardiac arrest. The heart was extracted and immersed in a container filled with University of Wisconsin Solution at 4°C for preservation.

Experimental Groups

The extracted hearts were preserved for 12 hours by the simple immersion method, under 2 different conditions: nonfreezing preservation at –3°C using CAS (the subzero group, n = 6) and conventional preservation at 4°C (the conventional group, n = 6). After preservation for 12 hours, each

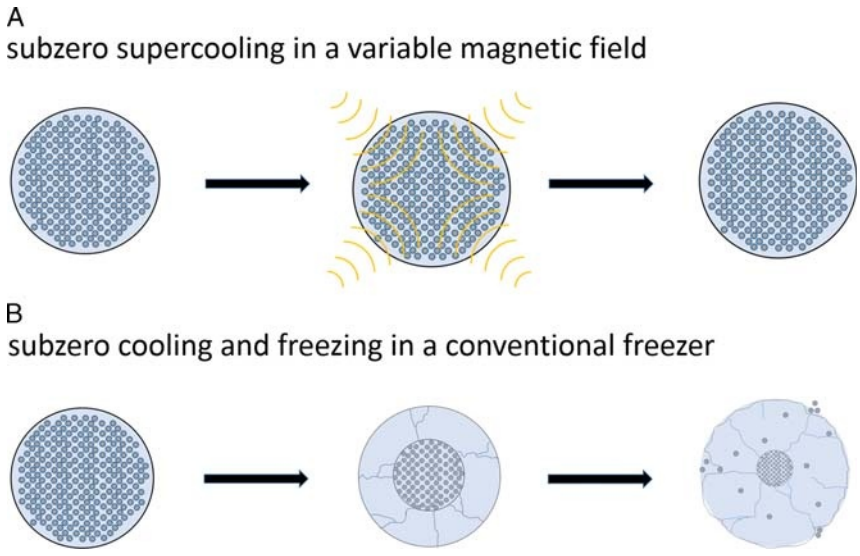

FIGURE 1. Schematic representation of cooling in a variable magnetic field. A, In the Cells Alive System, the organ is exposed to a variable magnetic field resulting in the oscillation of water molecules. Aggregation of water molecules is prevented and ice crystal formation is inhibited. The organ is maintained in a supercooled state. B, In a conventional freezer, freezing starts from the surface, and multilayered freezing occurs. Water molecules that cluster in the nonfrozen portion are transferred to the surface by capillary effect and cause rupture of cell membrane.[18]

heart was evaluated with respect to myocardial energy metabolism, ultrastructural alteration, and myocardial edema.

Subzero Supercooling System

For nonfreezing preservation at subzero temperature, the CAS was used. In this study, nonfreezing preservation was applied at –3°C, a temperature at which macroscopic freezing could not be observed.

Measurements of High-Energy Phosphate Level

A portion of the left ventricular myocardium was frozen using liquid nitrogen and weighed promptly before defrosting. Perchloric acid (0.6 N) that had been ice-cooled to 4°C was added at 2 mL per 0.2 g tissue and homogenized. The homogenate was centrifuged at 4°C, 3 000 rpm for 10 minutes. The supernatant was separated, and a neutralizing solution (triethanolamine potassium carbonate solution) was added at 0.1 mL per 1 mL supernatant, then stirred and left to stand for 10 minutes at 4°C. After centrifugation at 4°C, 3 000 rpm for 10 minutes, the supernatant was collected and the concentration of adenosine triphosphate (ATP) was measured by absorption spectroscopy (Lucifell 250 Plus; Kikkoman, Tokyo, Japan). The ATP concentrations are expressed in μmole per gram protein.

In addition to the 2 experimental groups, ATP concentration of 1 porcine heart was measured immediately after the heart was extracted from the pig for the control data.

Measurements of Tissue Lactate Concentration

Using the supernatant obtained after homogenization and centrifugation, lactate concentrations in the tissue were measured using the Determiner LA kit (Kyowa Medex Co. Ltd., Tokyo, Japan), an enzymatic assay using lactate oxidase. The lactate concentration was expressed in μmole per gram protein.

Measurements of Tissue Water Content

After heart preservation, the myocardium was sampled and weighed using a Classic Level analytical balance (Mettler Todedo, Tokyo, Japan) to measure the wet weight (WW). The tissue was then dried for 48 hours at 80°C and weighed again to measure the dry weight (DW). The water content (WC) in the myocardium was obtained using the following formula:

$$\mathrm{WC}\ (\%) = (\mathrm{WW} - \mathrm{DW})/\mathrm{WW} \times 100$$

Light Microscopy Experiments

Specimens of left ventricular free wall were extracted and fixed in 10% buffered formalin, dehydrated, embedded in paraffin, cut into sections, and mounted. After deparaffinization, the tissues were stained with periodic acid Schiff (PAS) for histopathologic evaluation of preserved glycogen.

Electron Microscopy Experiments

The subendocardium was extracted from the left ventricle and fixed in 2.5% glutaraldehyde. After washing with buffer solution, the tissue was fixed in 1% osmic acid, dehydrated with ethanol, processed with propylene oxide, and embedded in Epon. Ultrathin sections were cut using a diamond knife, stained with uranyl acetate, and observed under an electron microscope (field emission transmission electron microscope with high spatial and energy resolution HF-3300; Hitachi, Tokyo, Japan). From the electron microscopic images, mitochondrial functional scores were assessed according to Flameng et al.[19] Twenty mitochondria were randomly selected from images of each of 5 sections prepared for every sample (100 mitochondria in total). Each mitochondrion was scored on a scale of 0 to 4 using the following criteria: score 0, normal structure with preserved mitochondrial granules; score 1, normal structure but granules absent; score 2, swollen mitochondria with clearing of the matrix; score 3, disruption of mitochondrial cristae with clearing as well as condensation of the matrix; and score 4, disruption of the cristae and loss of integrity of the mitochondrial inner and outer membranes. Finally, the average score was calculated as the Flameng score (Figure 2).

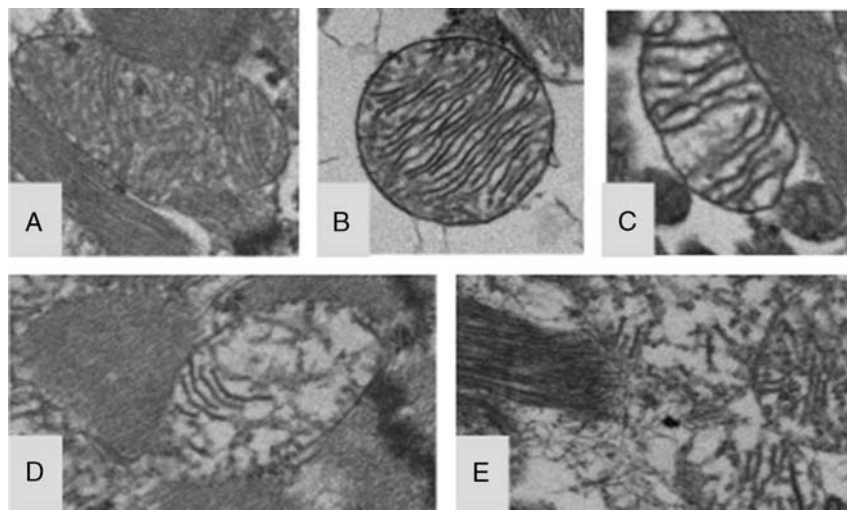

FIGURE 2. Postulated stages of mitochondrial changes after ischemic injury (Flameng score). A, Score 0: normal structure with preserved mitochondrial granules. B, Score 1: normal structure but granules absent. C, Score 2: swollen mitochondria with clearing of the matrix. D, Score 3: disruption of mitochondrial cristae with clearing and condensation of the matrix. E, Score 4: disruption of the cristae and loss of integrity of the mitochondrial inner and outer membranes.

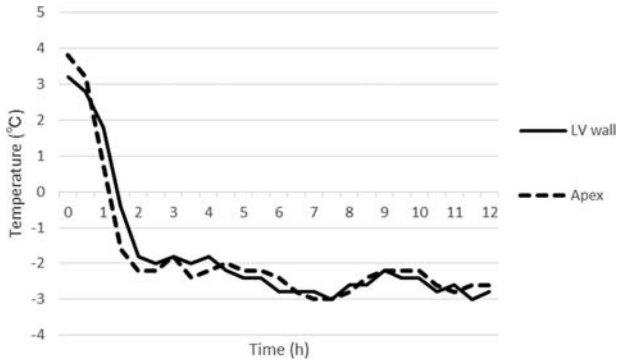

FIGURE 3. Myocardial temperature in a variable magnetic field (–3°C). The temperatures of the LV wall and apex were measured. LV indicates left ventricular.

Statistical Analysis

All experimental data are presented as mean ± standard deviation (M ± SD). SAS version 9.2 (SAS Institute, Carry NC, USA) was used for statistical analysis. A Mann-Whitney *U* test was performed, and a *P* value less than 0.05 was considered to be significant.

RESULTS

Nonfreezing Preservation

In a variable magnetic field, supercooling of the porcine heart was successfully maintained in at a temperature of –3°C (Figure 3).

Preservation of High-Energy Phosphate

The concentration of ATP in the left ventricular myocardial tissue immediately after the extraction was 18.04 μmol/g.

The concentration of ATP stored in the left ventricular myocardial tissue following the 12-hour preservation period was 21.06 ± 5.87 μmol/g in the subzero group and was significantly higher compared to 5.96 ± 3.41 μmol/g in the conventional group ($P < 0.05$) (Figure 4A).

Tissue Lactate Concentration

The concentration of lactate accumulated in the left ventricular myocardial tissue during the 12-hour preservation period was 6.51 ± 2.28 μmol/g in the subzero group and 11.15±3.74 μmol/g in the conventional group, suggesting a more efficient suppression of anaerobic metabolism at subzero temperatures ($P < 0.05$) (Figure 4B).

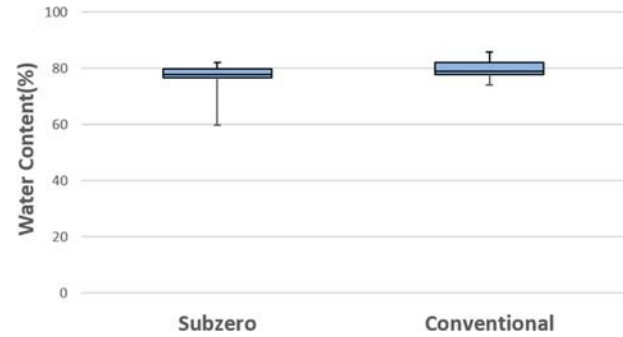

FIGURE 5. Tissue water content at the end of 12-hour preservation did not differ significantly between the subzero group and conventional group ($P = 0.39$).

Tissue Water Content

The difference in the tissue water content, which indicates the extent of myocardial edema, was not statistically significant (75.55±8.07% and 79.66±4.18%; $P = 0.39$) (Figure 5).

Histopathologic Findings in Light Microscopy

The PAS staining demonstrated that glycogen in myocytes was better preserved in the subzero group than in the conventional group (Figure 6).

Evaluation of Mitochondrial Structure in Electron Microscopy

In the subzero group, the structure of mitochondrial cristae and matrix were well preserved, and granules were observed within several mitochondria in 6 of 6 hearts. In the conventional group, clearing of the matrix was observed in 6 of 6 hearts, with disruption of the cristae in most mitochondria (Figure 7A). The Flameng score, which indicates the degree of mitochondrial impairment, was significantly lower in the subzero group compared to the conventional group (1.28±0.40 vs 2.73±0.30; $P < 0.05$) (Figure 7B).

DISCUSSION

In the present study, porcine hearts preserved using a subzero nonfreezing simple immersion method in a variable magnetic field at –3°C showed a well-preserved ultrastructure and better metabolic performance than those preserved using the conventional simple immersion method at 4°C. The novel simple, inexpensive, and reliable method with the use of CAS, which successfully preserved hemodynamics in

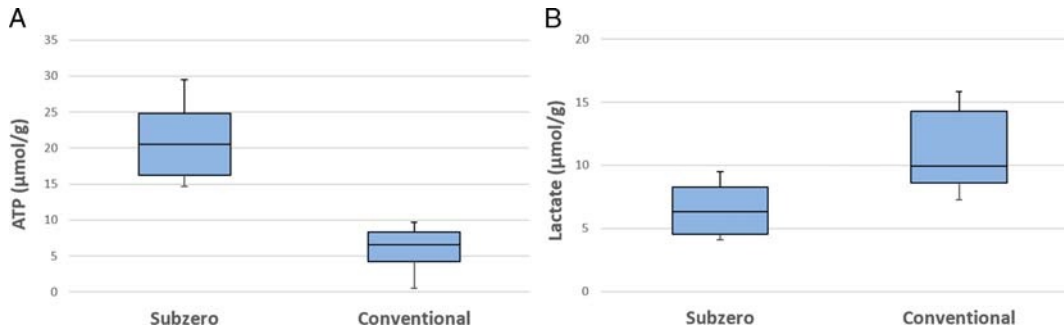

FIGURE 4. A, ATP levels in the anterior left ventricular wall after 12-hour preservation. The ATP level was significantly higher in the subzero group than in the conventional group ($P < 0.05$). B, Lactate levels in the anterior left ventricular wall after 12-hour preservation. The lactate level was significantly lower in the subzero group than in the conventional group ($P < 0.05$).

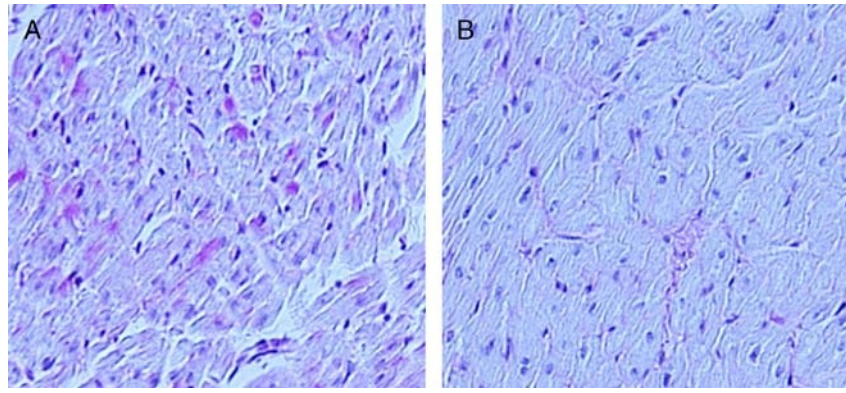

FIGURE 6. Histopathologic findings of light microscopy in the subzero group (A) and the conventional group (B). The intracytoplasmic glycogen is better preserved in the subzero group than in the conventional group (periodic acid-Schiff stain; original magnification, ×20).

rat hearts in a previous study,[18] showed better result in the fundamental experiment of preservation of porcine hearts.

Since 1967, the simple immersion method at 4 °C has been the standard method for preserving the donor heart.[20] For decades, the safe preservation time of donor hearts has not been extended beyond 6 hours. With the aim of expanding the donor pool, studies of coronary perfusion methods have been carried out in diverse institutes and numerous successful results have been reported for the preservation of animal hearts.[7-12] However, the clinical application of the coronary perfusion method has been held back by the technical complexity, concern over myocardial edema, and the cost of the apparatus.[20] On the other hand, studies of subzero nonfreezing cryopreservation by immersing the organ in a solution containing cryoprotectants were conducted in other institutes.[15-17] All the reports involved only small animals. Cryoprotectants in the solution mainly affect the surface of the cardiac tissue and are therefore difficult to apply to the preservation of large solid organs. Moreover, some cryoprotectants are reported to be toxic to the myocardium at a temperature of 37°C.[5] Therefore, a new strategy for subzero nonfreezing preservation has been anticipated.

Previously, several reports have demonstrated a reduction of cellular damage in a supercooled state through the

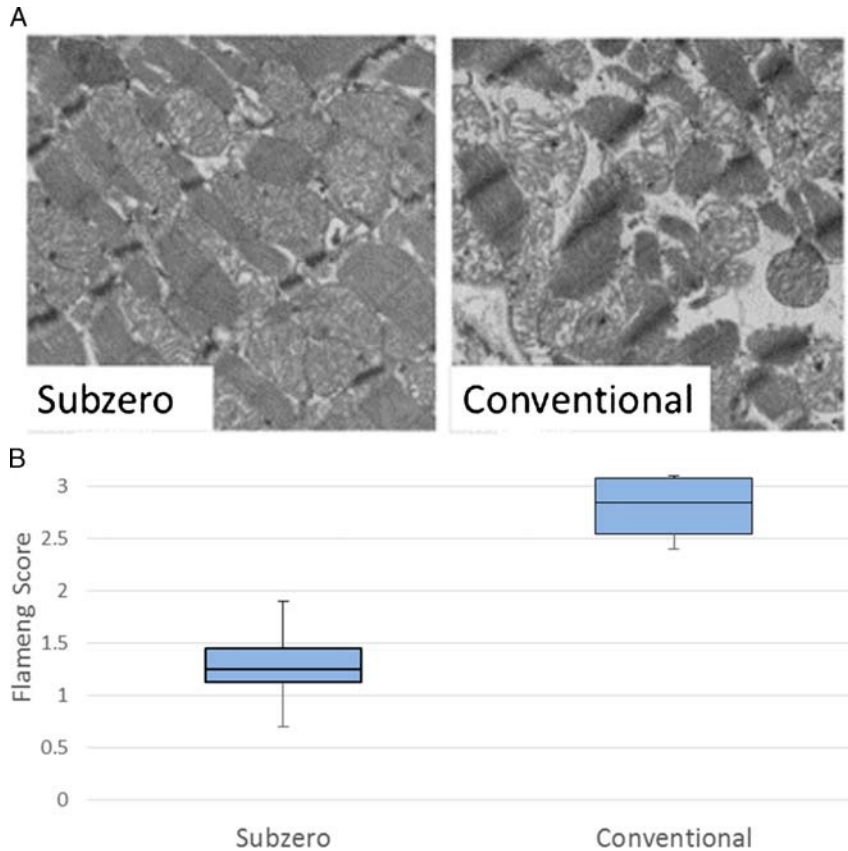

FIGURE 7. A, Subendocardial biopsy of the left anterior wall at the end of 12-hour preservation (×6 000). Subzero: the mitochondrial structures are well preserved in the subzero group. Conventional: mitochondria are swollen and clearing of matrix is observed in the conventional group. B, Flameng scores at the end of 12-hour preservation. The Flameng score was significantly lower in the subzero group than in the conventional group ($P < 0.05$).

application of magnetic fields. According to Okamoto et al,[21] when 3000 V is applied in preservation of a rat lung at a temperature of −2°C, reperfusion injury clearly declines compared to preservation at 4°C. Naito et al[22] reported that all *Drosophilia* survived under supercooled conditions with a magnetic field at 0°C for 72 hours, −4° C for 24 hours, and −8 °C for 1 hour; in contrast, all died without the magnetic field. These previous studies led us to focus on applying the CAS in the field of heart preservation. The CAS that generates a variable magnetic field was originally invented by a Japanese company, engaged in developing food-freezing technology, aiming to preserve fish and meat with little cellular damage and hence maintain freshness. Because the cellular damage due to coagulation is inhibited, foods could be preserved with less damage than in the conventional freezer. However, to apply the technology to the field of heart transplantation, we also had to avoid the cellular damage due to thawing. Once the material is frozen, the process of thawing is inevitable; thus, we focused on the ability of the variable magnetic field to keep materials in a nonfreezing state. In our experiment, the organ was preserved without macroscopic freezing at a temperature of −3°C. A variable magnetic field is generated in the entire machine, and every water molecule is simultaneously oscillated and cooled to subzero temperature. Thus, CAS makes nonfreezing preservation possible with large organs, such as porcine hearts.

The present study evaluated ATP and lactate concentrations in the myocardium after preservation. Intramyocardial ATP is essential for maintaining homeostasis and preventing irreversible damage to the organ during hypoxic storage.[3] Tissue lactate concentration during ischemia was reported by Neely et al[23] to be directly correlated with the decrease in the heart's ability to recover ventricular function with reperfusion. The intramyocardial energy generating system depends on 2 reactions, aerobic oxidative phosphorylation and anaerobic glycolysis. During ischemia, however, oxidative phosphorylation is suppressed and anaerobic glycolysis is used by the cells as an inefficient energy production mechanism. This results in a reduction in ATP and an increase in lactate, which is the end product of anaerobic glycolysis. Furthermore, the hydrogen ions generated by hydrolysis of ATP and the lowered intracellular pH due to lactate promote cell damage. The results of this study provide evidence that anaerobic metabolism is suppressed in porcine hearts preserved for 12 hours by immersion in a variable magnetic field at subzero temperature. Furthermore, the tissue lactate concentrations indicated that cardiac function after reperfusion can be expected to be better in a heart preserved in a variable magnetic field.

Histologic evaluation of light microscopy with PAS staining showed that glycogen in myocytes is well preserved in CAS. In a previous study, Nameki et al[11] carried out this method as an index of well-preserved myocytes. The persistence of glycogen also suggests that anaerobic metabolism is well suppressed in CAS.

In the present study, both qualitative assessment from electron micrographs and statistical evaluation of the Flameng scores demonstrated that ultrastructural mitochondrial damage in the subendocardium of the left ventricle was suppressed by subzero nonfreezing preservation in a variable magnetic field. Ultrastructural damage in myocardial tissue is caused by anaerobic metabolism, decreased pH, increased intracellular Ca, activation of phospholipase, destruction of phospholipids in the cellular membrane, and activation of intracellular protease. The sequential changes in myocardial tissue, including mitochondria, after ischemic injury were detailed by Jennings et al.[24] Ferrera et al[25] reported that mitochondria were the most sensitive subcellular components to prolonged hypothermia. Moreover, 1 report have indicated that the subendocardium of the left ventricle is most prone to damage by ischemia.[26] Because the most sensitive component of the tissue was well preserved in this area, the whole myocardium in the preserved heart can be expected to be intact.

Organ preservation in a magnetic field is a developing technology and has an infinite potential to revolutionize a transplantation medicine. The magnetic field not only inhibits the movement of water molecules but also has been reported to decrease the generation of reactive oxygen species and reduce the cellular damage.[27] Moreover, the magnetic field also has the potential to realize long-term freezing preservation of the heart. At temperatures below −60°C, metabolism in the cells is totally suppressed.[18] In a variable magnetic field, the growth of ice crystals inside and outside the cell is suppressed, and cellular injury due to the freezing process is prevented. So far, CAS has been applied to freezing cryopreservation of dental pulp tissues, dental pulp stem cells, embryonic stem cells, and mesenchymal stem cells.[28,29] If an advanced technique for suppressing cellular injury in the progress of thawing is invented in the future, the donor heart could be preserved in CAS with no damage for years and could be donated to recipients anytime anywhere in the world.

Study Limitations

The main limitation of this study was that a hemodynamic evaluation was not carried out after reperfusion. Based on our initial experience, we expect that successful results can be achieved regarding cardiac function after reperfusion after nonfreezing preservation compared to conventional preservation at 4°C. Before this novel method can be applied in clinical use, further research is necessary to evaluate the hemodynamic performance and long-term prognosis after transplantation.

CONCLUSIONS

The CAS showed remarkable performance in the suppression of anaerobic metabolism and tissue damage in porcine hearts.

ACKNOWLEDGMENTS

The authors thank Dr. Eisei Oda, Ph.D. for helpful cooperation in statistical analysis. The authors also thank ABI Co. Ltd. (Chiba, Japan) for the application of CAS in support of this study.

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